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(54) Title: NON–HOMOGENEOUS SYSTEMS FOR THE RESOLUTION OF ENANTIOMERIC MIXTURES

(57) Abstract

The present invention relates to a process for the biocatalyst–mediated enantioselective conversion of enantiomeric mixtures of hydrophobic esters using a biphasic solvent system. More particularly, the present invention relates to the enzyme–mediated enantioselective synthesis of anti–viral compounds, such as 2–hydroxymethyl–5–(5–fluorocytosin–1–yl)–1,3–oxathiolane (FTC) and its analogues, in a non–homogeneous reaction system.

## NON-HOMOGENEOUS SYSTEMS FOR THE
## RESOLUTION OF ENANTIOMERIC MIXTURES

### TECHNICAL FIELD OF THE INVENTION

The present invention relates to a process
5   for the biocatalyst-mediated enantioselective
conversion of enantiomeric mixtures of hydrophobic
esters using a biphasic solvent system.  More
particularly, the present invention relates to the
enzyme-mediated enantioselective synthesis of anti-
10  viral compounds, such as 2-hydroxymethyl-5-(5-
fluorocytosin-1-yl)-1,3-oxathiolane (FTC) and its
analogues, in a non-homogenous reaction system.

### BACKGROUND OF THE INVENTION

Serious obstacles to commercially viable
15  processes for the enzymatic resolution of enantiomeric
mixtures of hydrophobic esters exist.  For example,
when using an enzymatic conversion process in the
presence of an organic solvent, the rate of enzyme
inactivation is very high relative to the same process
20  performed in an aqueous solvent.  A confounding problem
is that solvents which are less destructive to the
catalyst are often less able to solubilize the more
hydrophobic substrates.  Ideally, many processes would

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be more efficient if they were performed in more
hydrophobic solvents, such as non-miscible organic
solvents.  One goal of the present invention is to
provide a non-homogenous system, which allows higher
5   concentrations of hydrophobic substrates to be
converted to product, while simultaneously consuming
less catalyst.

      The above-cited obstacles must be overcome in
order to reduce the cost of producing enantiomeric
10  drugs anti-viral drugs.  Such drugs are vital towards
winning the struggle to conquering emerging viral
diseases.  For example, even today, the rate of HIV
infection continues at a staggering pace, with 16,000
new infections per day worldwide [Balter, M. Science
15  280, 1863-1864 (1998)].  There are areas of sub-Saharan
Africa where at least 25% of the population are
infected, for example in Botswana and Zimbabwe.  The
cost of anti-viral drugs, however, is currently far
beyond the reach of most such victims of HIV infection.

20        Nucleoside analogues, such as 3'-
thiaribofuranonsyl-βL-cytosine ("3-TC"), 3'-azido-3'-
deoxythymidine (AZT) [Blair E., Darby, G., Gough, E.,
Littler, D., Rowlands, D., Tisdale, M. *Antiviral
Therapy*, BIOS Scientific Publishers Limited, 1998],
25  (-)-2',3'-dideoxy-5-fluoro-3'-thiacytidine ("FTC") and
2',3'-dideoxy-3'-thiacytidine are important antiviral
agents [Liotta, D.C. 216th ACS National Meeting,
Medicinal Chemistry Abstract, Boston, MA, August 2327,
1998; Hoong, L.K., Strange, L.E., Liotta, D.C.,
30  Koszalka, G.W., Burns, C.L., and Schinazi, R. F., *J.
Org. Chem.* 1992, 57, 5563-5565].  3-TC has been
marketed as both an anti-HIV and an anti-HBV drug and
FTC is under clinical trial for evaluation as an anti-

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viral drug [Liotta, D.C., Schinazi, R.F., and Choi, W.-
B., United States patents 5,210,085,  5,700,937 and
5,814,639].  Since it is the (-) enantiomer of both (-
)-FTC and (-)-2',3'-dideoxy-3'-thiacytidine, which

5   exhibits the most potent anti-viral activity and the
least toxicity, as compared to the corresponding (+)-
isomers, there is a pressing need for efficient cost-
effective methods of preparation of both the (-)-FTC
and (-)-2',3'-dideoxy-3'-thiacytidine isomers to expand

10  treatment options of patients throughout the world
[Liotta, D.C. 216th ACS National Meeting, Medicinal
Chemistry Abstract, Boston, MA, August 23-27, 1998;
Hoong, L.K., Strange, L.E., Liotta, D.C., Koszalka,
G.W., Burns, C.L., and Schinazi, R.F., *J. Org. Chem.*

15  1992, 57, 5563-5565].
        Many hydrolase enzymes have been used for the
resolution of FTC esters [Hoong, L.K., Strange, L.E.,
Liotta, D.C., Koszalka, G.W., Burns, C.L., and
Schinazi, R. F., *J. Org. Chem.* 1992, 57, 5563-5565].

20  Impediments remain, however, to developing practical
enzyme mediated chemical processes for the production
of FTC and similar compounds.  First, the solubility of
many FTC esters in aqueous media is too low to achieve
economically viable production of resolved product.

25  One possible solution has been to add a water miscible
co-organic solvent to increase the concentration of the
ester in solution.  An example is the use of solutions
of acetonitrile and water [Hoong, L.K., Strange, L.E.,
Liotta, D.C., Koszalka, G.W., Burns, C.L., and

30  Schinazi, R.F., *J. Org. Chem.* 1992, 57, 5563-5565;
Liotta et al., United States patent 5,827,727].
Although the use of a water miscible organic solvent
and water solution increases the concentration of

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substrate in solution, it has the unfortunate effect of
drastically lowering the enzyme catalyzed conversion
and enzyme stability.  This problem is especially
pronounced, where the substrate is not completely
5    dissolved, but is also present as an undissolved solid
suspension (high concentration of substrate loading).
Similar results were obtained in our laboratory.  When
water miscible organic solvents, such as isopropanol,
dimethylformamide (DMF), 1-methyl-2-pyrrolidinone,
10   dimethylsulfoxide (DMSO), methanol, acetonitrile,
ethanol, 1-propanol were used as co-solvent for the
resolution, the maximal substrate concentration loading
was 3%.  The presence of undissolved substrate
decreased the enantioselectivity when the substrate
15   concentration was beyond 3%.  Furthermore, use of a
water miscible organic solvent and water solution, at
concentrations of water miscible organic co-solvents of
greater than 20%, had a pronounced negative impact on
enzyme activity, especially for porcine liver esterase
20   (PLE).

The present invention specifically addresses
several obstacles in the art that had the effect of
making enzymatic resolution of enantiomeric mixtures
uneconomical.  First, it was thought that enzymatic
25   conversion should be performed under homogenous
conditions, because biphasic systems result in poor
reproducibility [See Liotta et al., United States
patents 5,827,727, 5,892,025, 5,914,331].  One
potential advantage for the use of non-homogenous
30   systems would be in enhanced solubilization of the
substrate.  Presumably, in a non-homogenous system, a
higher concentration of many hydrophobic substrates
could be accommodated.  Prior to the present invention,
it was believed that alcohol solvents should be

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avoided, because these solvents denature enzymes [Liotta et al., United States patents 5,827,727, 5,892,025, 5,914,331]. The present invention is an advance over the art because it specifically provides

5   for the use of alcohol solvents which form non-homogenous systems with water. In addition, the use of non-homogenous solvent systems provides increased solubilization of more hydrophobic substrates than could be accomodated previously in the art.

10  Furthermore, the present invention discloses a process which requires less enzyme per unit of product.

Additional improvements achieved via the present invention permit the use of several alcohol solvents in an enzymatic process. In addition, the

15  present invention provides an alternative process mode, wherein enzyme and organic solvent requirements are further reduced by the addition of surfactants. Finally, the present invention is directed to providing a more efficient enzymatic process which maintains the

20  enantioselectivity at a high level.


## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the enantioselective conversion of one enantiomeric form of an enantiomeric mixture of FTC butyrate to the corresponding non-

25  racemic alcohol and the desired non-racemic ester.


## SUMMARY OF THE INVENTION

The present invention is directed to several improvements in processes for producing a chiral, non-racemic ester. More specifically, the present

30  invention is directed to providing an improved process

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that uses a biphasic non-homogenous system employing biocatalysts to resolve enantiomeric mixtures of FTC esters and analogues of FTC esters. The invention is further directed to improvements which allow high·
5  substrate loading and consume reduced amounts of enzyme.

A first improved process according to this invention provides for dispersing an enantiomeric mixture of an ester in an organic solvent system to
10  produce an organic component at a high substrate loading. An aqueous component is provided and preferentially contains a dispersed hydrolase enzyme. Alternatively, the hydrolase enzyme can be added to the entire non-homogenous system or less preferentially to
15  the organic component. The process further requires contacting the organic component and the aqueous component to form a non-homogenous system, under conditions which permit the resolution of the mixture to produce a chiral non-racemic ester and a non-racemic
20  alcohol. The combination of the organic component and aqueous component form a non-homogenous system. ·By using a non-homogenous system, much higher substrate concentrations are possible. In one embodiment, after the reaction is carried out, the chiral non-racemic
25  ester compound may be isolated from the organic component and the chiral non-racemic alcohol compound may be isolated from the aqueous component. The isolation steps may vary, depending on the particular compound and conditions.
30            This invention also provides an alternative process that produces improved results by drastically reducing the amount of enzyme required for producing a given product. Such improvement is achieved by the addition of surfactant to said non-homogenous system,

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to produce an improved non-homogenous system that requires less organic solvent to solubilize the substrate.

In another embodiment, the invention provides
5 a process using a lowered organic/water phase ratio, which results in a further reduction in the required hydrolase enzyme.

In another embodiment of this invention, addition of surfactant to the system permits
10 enhancement of enzyme reaction rates and better solubilization of substrate. Higher rates of reaction result in a lower overall enzyme costs for operating the process.

## DETAILED DESCRIPTION OF THE INVENTION

15 In the following description, terms are defined as follows:

Biocatalyst -- a protein molecule, such as a hydrolase enzyme. Examples include esterases, proteases and lipases.

20 Chiral compound -- a compound that is not superposable on its mirror image, and usually contains an asymmetric carbon atom, where four different groups are attached to the same carbon.

Co-solvent -- an organic solvent.

25 Conversion -- the process of treating an enantiomeric mixture of compounds with a catalyst which transforms a single enantiomer into a different chemical entity.

Diastereomers -- stereoisomers that are not
30 related as mirror reflections of one another.

Dispersing -- distributing the enzyme or enantiomeric mixture material in the solvent. The

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enzyme may be in the form of a crosslinked enzyme
crystal, immobilized enzyme, or soluble enzyme, and the
enantiomeric mixture may be soluble or contain residual
particulates. The disperse system may contain up to
5   three phases with solid crystalline and/or particulate
materials and two different liquid phases.

Enantiomers -- pairs of stereoisomers that
are mirror reflections of each other. An enantiomer is
non-superposable on its mirror image. Enantiomers are
10  chiral stereoisomers that differ only in how they react
with other chiral molecules and in their behavior
toward plane polarized light. Separate enantiomers
rotate the plane of polarized light in equal but
opposite directions. Different enantiomers are
15  distinguished by the R and S designations and whether
the plane of polarized light is rotated to the right
(dextrorotary (+)) or to the left (levorotatory (-)).

Enantiomeric excess -- in a mixture
(solution) of two enantiomers where one enantiomer is
20  present to a greater extent, the solution will display
optical rotation (+ or - rotation) corresponding to the
enantiomer which is present in excess. Enantiomeric
excess is the percentage of the enantiomer found in
excess over that of the racemic mixture and is
25  calculated as follows:

     (specific rotation of the mixture) ÷ (specific
     rotation of the pure enantiomer) X 100 = %
     enantiomer excess.

Enantiomeric mixture -- a mixture of two
30  enantiomers.

Enantioselectivity -- a preference for
converting one enantiomer from an enantiomeric mixture.

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FTC butyrate -- refers to an enantiomeric
mixture of the compound 2',3'-dideoxy-5'-butyrate-5-
fluoro-3'-thiacytidine or, using alternative
nomenclature, the compound is 2-butyryloxymethyl-5-(5-
5    fluorocytosin-1-yl)-1,3-oxathiolane or, less formally
the 5' butyrate ester of 2-hydroxymethyl-5-(5-
fluorocytosin-1-yl)-1,3-oxathiolane.

Incompletely water-miscible organic solvent
-- an organic solvent which is not fully soluble in
10   water at 25°C and forms non-homogenous solutions with
water.  not completely

Non-homogenous system - - a biphasic medium
comprising a biocatalyst, organic component, aqueous
component and a substrate.  A non-homogenous system may
15   also be referred to as a non-homogenous medium or a
non-homogenous condition or a non-homogenous
composition.

Organic solvent system -- a solution
comprising one or more of the following solvents: $C_1-C_8$
20   unsubstituted alkanes, alcohols, aromatics, ketone
ethers, nitro, halo-alkane or -aromatic organic
solvent, such as tert-amyl alcohol, iso-amyl alcohol,
1-pentanol, 3-pentanol, 1-butanol, 2-butanol, tert-
butanol, 3-methyl-3-pentanol, 4-methyl-2-pentanol, 3-
25   ethyl-3-pentanol, 3-heptanol, toluene, butylacetate,
nitroethane, nitromethane, dichloromethane, methyl
isobutyl ketone, dimethyl sulfide, sulfolane or any
other not more than about 50% water miscible organic
solvent which facilitates the dissolution of an
30   enantiomeric mixture without destroying the enzyme's
ability to function.

Racemic mixture -- an equimolar mixture of
two enantiomers, also known as a racemic modification,
usually produced as a result of a chemical reaction at

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a chiral center where neither enantiomeric product is preferred.

Resolving enantiomers or resolution -- the process of separating pairs of enantiomers from an
5 enantiomeric mixture.

Resolution of a racemic mixture -- the separation of a racemic mixture of enantiomers.

Stereochemistry of FTC and FTC Butyrate -- The stereochemistry of the FTC compounds referred to
10 throughout this application are shown below:

(-)-FTC Butyrate       --      C_3H_7COO

(-)-FTC       --       HO

+)-FTC Butyrate       --      C_3H_7COO

+)-FTC       --       HO
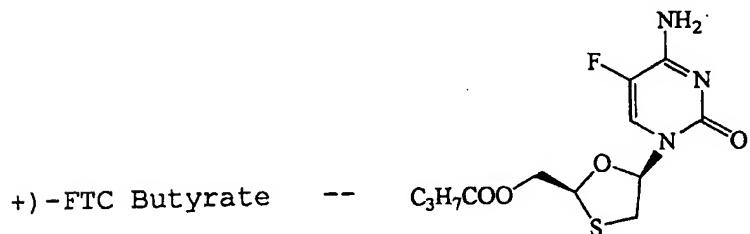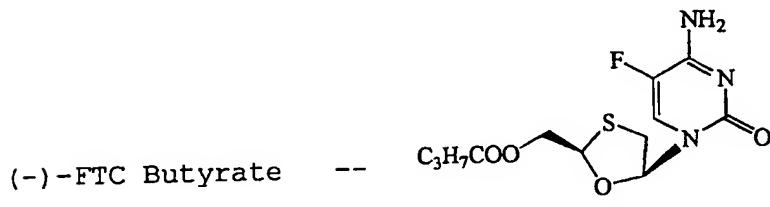
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Stereoisomer -- a compound whose constituent atoms are arranged in the same order as that of another compound, but differ only in the arrangement of their atoms in space.  Examples of stereoisomers are

5 enantiomers and diastereomers.

Substrate loading -- the concentration of an enantiomeric mixture.  For the examples shown below, substrate loading is expressed as % (weight/volume of the non-homogenous system), i.e., based on total

10 solvent volume.  To reiterate, percentage (%) (weight/volume) substrate loading is based on the volume of the entire non-homogenous system, which includes both the aqueous and organic components.

Surfactant -- surface active agents that

15 reduce the surface tension of solutions when dissolved in said solutions.  Surfactants also reduce the interfacial tension between two liquids, or between a liquid and a solid.  Surfactants belong to three categories which function through a similar mechanism.

20 Those categories include detergents, emulsifiers and wetting agents depending on the nature of the surfaces involved.  The surfactant concentration is expressed as percentage (%) (weight/volume) and is based on the volume of the entire non-homogenous system, which

25 includes both the aqueous and organic components.

Water-immiscible organic solvent -- an organic solvent which has a maximum solubility in water of 10% at 25°C and forms non-homogenous solutions with water.  The organic solvent concentration is expressed

30 as percentage (%) (volume/volume) and is based on the volume of the entire non-homogenous system, which includes both the aqueous and organic components.

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Not more than about 50% water-miscible
organic solvent -- an organic solvent which is not more
than about 50% soluble in water at 25°C and forms a
non-homogenous solution with water.

5        Water-miscible organic co-solvent -- an
organic solvent which is fully miscible in water at
25°C.

The present invention provides a process for
producing a chiral, non-racemic ester of Formula I
10   using a hydrolase enzyme:
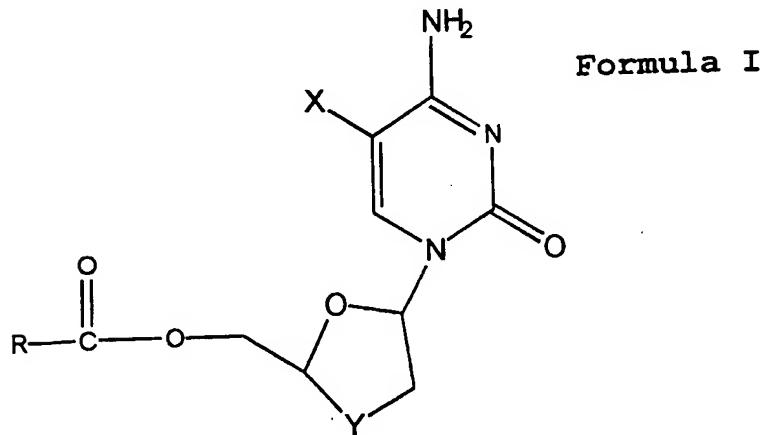


**Formula I**

wherein:
R is $C_1$-$C_8$ alkyl, alkenyl, or alkynyl;
15       X = H, or F;
Y = $CH_2$, O, S, Se, or NH;
said process comprising the steps of:
(a) dispersing an enantiomeric mixture of an
ester of Formula I at a concentration of between about
20   1 and about 25% (weight/volume of the non-homogenous
system), in an organic solvent system to produce an
organic component;

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(b) providing an aqueous solvent system to produce an aqueous component; and

(c) contacting said organic component and said aqueous component to form a non-homogenous system,
5 under conditions which permit the resolution of the mixture to produce a chiral non-racemic ester of Formula I and a non-racemic alcohol of Formula II;

**Formula II**
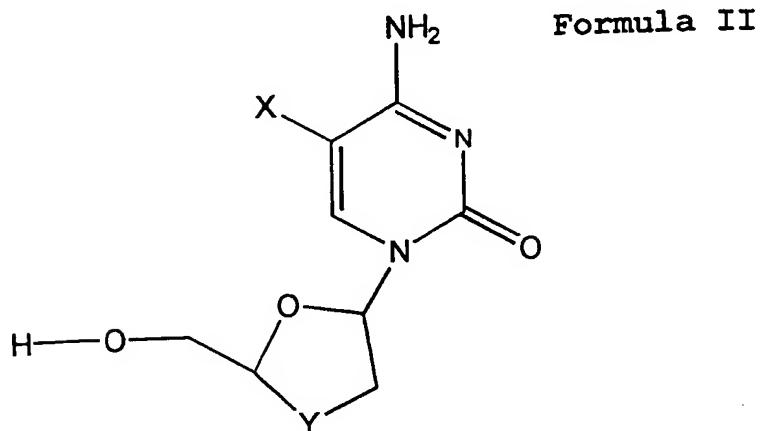


wherein:
10 X = H, or F;
Y = CH$_2$, O, S, Se, or NH, and
wherein said hydrolase enzyme is dispersed in either said organic component, said aqueous component or said non-homogenous system.

15 The present invention also provides a process for producing a chiral, non-racemic hydrophobic ester using a hydrolase enzyme, said process comprising the steps of:

(a) dispersing an enantiomeric mixture of
20 said hydrophobic ester at a concentration of between about 1 and about 25% (weight/volume of the non-

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homogenous system), in an organic solvent system to produce an organic component;

(b) providing an aqueous solvent system to produce an aqueous component; and

5    (c) contacting said organic component and said aqueous component to form a non-homogenous system, under conditions which permit the enantioselective conversion of one enantiomeric form of said enantiomeric mixture to the corresponding alcohol; and

10    wherein said hydrolase enzyme is dispersed in either said organic component, said aqueous component or said non-homogenous system.

Alternatively, the present invention provides processes for producing a chiral, non-racemic ester of

15  Formula I from an enantiomeric mixture of formula I or from an enantiomeric mixture of a hydrophobic ester, wherein said process further comprises a surfactant.

In addition, the present invention provides a process for producing a chiral, non-racemic ester of 2-

20  butyryloxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane using a hydrolase enzyme, said process comprising the steps of:

(a) dispersing an enantiomeric mixture of said 2-butyryloxymethyl-5-(5-fluorocytosin-1-yl)-1,3-

25  oxathiolane at a concentration of between about 1 and about 25% (weight/volume of the non-homogenous system), in an organic solvent system to produce an organic component;

(b) providing an aqueous solvent system to

30  produce an aqueous component; and

(c) contacting said organic component and said aqueous component to form a non-homogenous system,

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under conditions which permit the enantioselective
conversion of one enantiomeric form of said
enantiomeric mixture to the corresponding alcohol;
                      wherein said hydrolase enzyme is
5   dispersed in either said organic component, said
aqueous component or said non-homogenous system; and
                      wherein the concentration of said
enantiomeric mixture is calculated based on the volume
of said non-homogenous system.


10          One embodiment of this invention provides a
process for producing a chiral, non-racemic ester of 2-
butyryloxymethyl-5-(5-fluorocytosin-1-yl)-1,3-
oxathiolane using a hydrolase enzyme, said process
comprising the steps of:
15          (a) dispersing an enantiomeric mixture of
said 2-butyryloxymethyl-5-(5-fluorocytosin-1-yl)-1,3-
oxathiolane at a concentration of between about 1 and
about 25% (weight/volume of the non-homogenous system),
in an organic solvent system to produce an organic
20  component;
                      (b) providing an aqueous solvent system to
produce an aqueous component; and
                      (c) contacting said organic component and
said aqueous component to form a non-homogenous system,
25  under conditions which permit the enantioselective
conversion of one enantiomeric form of said
enantiomeric mixture to the corresponding alcohol;
                      wherein said hydrolase enzyme is
dispersed in either said organic component, said
30  aqueous component or said non-homogenous system;
                      wherein said organic component comprises
between about 5 and about 90% of said non-homogenous
system;

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wherein said non-homogenous system also comprises between about 1 and about 20% of surfactant; and

wherein said surfactant concentration is
5  calculated based on the volume of said non-homogenous system.

Another object of the present invention is to provide a non-homogenous system for producing a chiral, non-racemic hydrophobic ester using a hydrolase enzyme,
10  comprising:

(a) a hydrolase enzyme;

(b) a hydrophobic ester substrate;

(c) an organic component; and

(d) an aqueous component.

15  It is an object of this invention to provide a process for resolving a desired enantiomer from an enantiomeric mixture.

It is also an object of this invention to provide a process for resolving a desired enantiomer
20  from an enantiomeric mixture of hydrophobic esters.

It is a further object of this invention to provide a process for resolving enantiomers of anti-viral compounds having Formula I above.

The most preferred embodiment of this
25  invention provides a process for resolving enantiomeric FTC butyrate (or where R is propyl, X = F and Y = S of compound Formula I above).

Substrate loading entails dispersing an enantiomeric mixture of a hydrophobic ester in an
30  organic solvent system to produce an organic component. The concentration range expressed in units of % (weight/volume of the non-homogenous system) is selected

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from the group consisting of ranges between about 0.5%
and about 45%; between about 1.0% and about 45%;
between about 5.0% and about 45%; between about 10% and
about 40%; between about 10% and about 30%; between

5    about 5 and about 20%; between about 1% and about 5%;
and between about 10% and about 20%.

In a preferred embodiment, the organic
solvent systems of this invention, comprise one or
more, not more than about 50% water miscible organic

10   solvents, that facilitate dissolution of the
enantiomeric mixture.
In the next preferred embodiment, the organic
solvent systems of this invention, comprise one or
more $C_4$-$C_8$ alcohols.

15       In the most preferred embodiment, the organic
solvent systems of this invention, comprise one or both
of n-amyl alcohol or 3-methyl-3-pentanol.

In a preferred embodiment, the aqueous
solvent systems of this invention comprise water, one

20   or more buffering salts, alkalizing agents,
antimicrobial preservatives, stabilizers, filtering
aids, co-enzymes, or other excipients that facilitate
dispersion and function of the enzyme.
In the next preferred embodiment, the aqueous

25   solvent systems of this invention comprise water, one
or more buffering salts, alkalizing agents, or other
excipients that facilitate dispersion and function of
the enzyme.
In a next preferred embodiment, the aqueous

30   solvent systems of this invention comprise water, and
between about 0.01 and about 0.5 molar phosphate buffer
at a pH of between about 7.0 and about 8.0.

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In the most preferred embodiment, the aqueous solvent systems of this invention comprise water, between about 0.2 and about 0.4 molar phosphate buffer at a pH of between about 7.2 and about 7.8.

5          In another embodiment of this invention, the hydrolase enzyme is capable of resolving a pair of enantiomers.

In another embodiment of this invention, the hydrolase enzyme is capable of resolving a pair of
10    enantiomers by an enzyme catalyzed stereoselective reaction with one enantiomer.

In a preferred embodiment of this invention, the hydrolase enzyme is capable of resolving a pair of enantiomers by an enzyme catalyzed stereoselective
15    conversion of one enantiomer.

In the most preferred embodiment of this invention, the hydrolase enzyme is capable of resolving a pair of enantiomers by the enzyme catalyzed stereoselective conversion of the (+) enantiomer of 2-
20    butyryloxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane (or where R is propyl, X = F and Y = S of Formula I above or FTC butyrate).

In one embodiment of this invention, the biocatalyst is an enzyme.

25          In another preferred embodiment of this invention, the enzyme is a hydrolase.

In a preferred embodiment of this invention, the enzyme is selected from the group consisting of esterases, lipases and proteases.

30          In the most preferred embodiment of this invention, the enzyme is selected from the group consisting of porcine pancreatic lipase ("PL"),

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*Pseudomonas species* lipase, *Aspergillus niger* lipase, subtilisin, or porcine liver esterase ("PLE").

In one embodiment of this invention, the biocatalyst is added to the non-homogenous system after
5   the aqueous component is contacted with the organic component to make a non-homogenous system.

In another embodiment of this invention, a biocatalyst is added to the organic phase as part of the  organic component before the aqueous component is
10  contacted with the organic component to make a non-homogenous system.

In a preferred embodiment of this invention, a biocatalyst is added to the aqueous phase to create an aqueous component after the aqueous component is
15  contacted with the organic component but before agitation and mixing to make a non-homogenous system.

In the most preferred embodiment of this invention, a biocatalyst is added to the aqueous phase to create an aqueous component before the aqueous
20  component is contacted with the organic component to make a non-homogenous system.

In one embodiment of this invention, the non-homogenous system used in the process to resolve enantiomeric mixtures contains surfactant.  The
25  concentration range of surfactant in % (weight/volume of the non-homogenous system) is selected from the group consisting of between about 1 and about 30% of surfactant; between about 1% and about 20% of surfactant; between about 1% and about 10% of
30  surfactant; between about 1% and about 5% of surfactant; between about 5% and about 30% of surfactant; between about 10% and about 25% of surfactant; between about 15% and about 25% of

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surfactant; between about 20% and about 30% of
surfactant; and between about 5% and about 15% of
surfactant.

 In one embodiment of this invention, the
5    enzyme is immobilized on a matrix.

 In a preferred embodiment of this invention,
the enzyme form is that of a crosslinked enzyme
crystal, such as, for example, those described in PCT
patent application WO 92/02617 (Navia *et al.*).

10    In the next preferred embodiment of this
invention, the enzyme form is that of a controlled
dissolution crosslinked protein crystal, such as, for
example, those described in PCT patent application WO
98/46732 (Margolin *et al.*).

15    In the most preferred embodiment of this
invention, the enzyme is in a soluble form.


 In one embodiment of this invention, said
non-homogenous systems comprise between about 10% and
99% organic component. In another embodiment of this
20   invention, said non-homogenous systems comprise between
about 10% and about 90% organic component. More
preferably non-homogenous systems comprise between
about 20% and about 80% organic component. Even more
preferably, said non-homogenous systems comprise
25   between about 30% and about 70% organic component. In
an even more preferred embodiment, said non-homogenous
systems comprise between about 10% and about 50%
organic component. In another preferred embodiment,
said non-homogenous systems comprise between about 10%
30   and about 60% organic component. In a further
preferred embodiment, said non-homogenous systems
comprise between about 20% and about 70% organic
component. In still another preferred embodiment, said

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non-homogenous systems comprise between about 50% and about 20% organic component.

   In one embodiment of this invention, said processes for resolving a desired enantiomer are
5  carried out at a temperature or temperatures selected from the group consisting of between about 0°C and about 45°C; between about 10°C and about 45°C; between about 20°C and about 45°C; between about 30°C and about 45°C; between about 10°C and about 40°C; between about
10 10°C and about 30°C; between about 10°C and about 25°C; between about 15°C and about 40°C; between about 15°C and about 35°C; between about 15°C and about 30°C; between about 15°C and about 25°C; and between about 20°C and about 35°C

15    In a preferred embodiment, said aqueous component used in the processes of this invention comprises at least 10% (volume/volume) of said non-homogenous system.
   In the next preferred embodiment, said
20 aqueous component used in the processes of this invention comprises at least 50% (volume/volume) of said non-homogenous system.
   In the most preferred embodiment, said aqueous component used in the processes of this
25 invention comprises at least 90% (volume/volume) of said non-homogenous system.
homogeneous

   In one embodiment of this invention, said process for resolving a desired enantiomer is carried
30 out in a non-homogeneous system comprising a surfactant. When a surfactant is part of said non-

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homogeneous system, the concentration range of the
organic component in %(volume/volume) is selected from
the group consisting of between about 5% and about 90%
of said non-homogeneous system; between about 5% and

5    about 80% of said non-homogeneous system; between about
5% and about 70% of said non-homogeneous system;
between about 5% and about 60% of said non-homogeneous
system; between about 5% and about 50% of said non-
homogeneous system; between about 5% and about 30% of

10   said non-homogeneous system; between about 5% and about
20% of said non-homogeneous system; between about 5%
and about 10% of said non-homogeneous system; between
about 10% and about 30% of said non-homogeneous system;
between about 10% and about 20% of said non-homogeneous

15   system; between about 20% and about 70% of said non-
homogeneous system; or between about 25% and about 50%
of said non-homogeneous system; and between about 30%
and about 60% of said non-homogeneous system.

        The reaction scheme for resolution of an

20   enantiomeric mixture is illustrated in the reaction
shown in Figure 1 (infra), where the substrates were
either, acetate, formate, propionate, butyrate,
pentanoate or other n-alkyl and branched chain or aryl
esters of FTC, or derivatives of such esters of FTC and

25   the organic co-solvents were any that werenot more than
about 50% water miscible alcoholic, alkane, aromatic,
ketone ether, nitro, halo-alkane or aromatic organic
solvents, such as n-amyl alcohol, iso-amyl alcohol,
tert-amyl alcohol, 3-pentanol, 1- or 3-heptanol, 3-

30   methyl-3-pentanol, 4-methyl-2-pentanol, 3-ethyl-3-
pentanol, 1- or 2-butanol, nitromethane,
dichloromethane, methyl isobutyl ketone, dimethyl
sulfide, sulfolane, and others.

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In Figure 1, shown below, the products of the
reaction were a non-racemic ester and a non-racemic
alcohol (Figure 1). In one example, when X is
Fluorine, R is $C_3H_7$ and Y is Sulfur, then compound A
5   represents an enantiomeric mixture of FTC butyrate.
Various hydrolytic enzymes such as, porcine liver
esterase (PLE), lipase from *Pseudomonas species* (PSL)
and lipase from *Aspergillus niger* (ANL) have been used
as catalyst [For PLE catalyzed reactions in mixed
10  organic solvents: *See* Ariente-Fliche, C., Braun, J.,
and Le Goffic, F., *Synth. Commun.* 22, 1149-1153 (1992);
Basavaiah, D., and Krishna, P.R., *Pure & Applied Chem.*,
64, 1067-1072 (1992); Basavaiah, D., Pandiaraju, S.,
and Muthukumaran, K., *Tetrahedron: Asymmetry,* 7, 13-16,
15  (1996); Mahmoudian, M., Baines, B.S., Dawson, M.J., and
Lawrence, G.C., Enzyme Microb. Technol., 14, 911-916,
(1992); Izumi, T. and Kasahara, A., Japanese patent
JP08092269A (1996)].

**Figure 1**
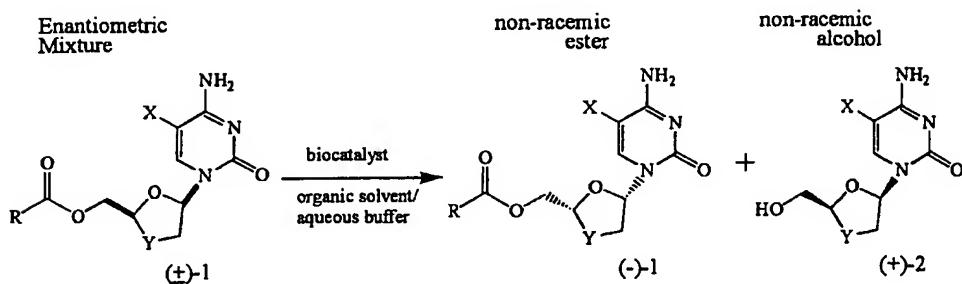


R is $C_1-C_8$ alkyl, alkenyl, or alkynyl; X = H,
20  or F; Y = $CH_2$, O, S, Se, or NH; the
biocatalyst can be either soluble enzyme,
immobilized, or the cross-linked enzyme
crystal form; the organic co-solvent can be

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any that were not more than about 50% water
miscible organic solvents, such as n-amyl
alcohol, *iso*-amyl alcohol, *tert*-amyl alcohol,
3-pentanol, 1- or 3-heptanol, 3-Me-3-

5      pentanol, 4-Me-2-pentanol, 3-Et-3-pentanol,
1- or 2-butanol, nitromethane,
dichloromethane and others.


The biocatalysts may be either soluble enzyme,
immobilized enzyme or crosslinked crystal (CLEC™) form

10    of the enzyme (Altus Biologics, Inc., Cambridge,
Massachusetts). The reaction can be performed in a
batch reactor, a column, a hollow-fiber membrane
[Enzyme Catalysis in Organic Synthesis, pp. 138-150,
edited by Drauz, K. and Waldmann, H., VCH

15    Verlagsgesellschaft GmbH, Weinheim, 1995] or membrane
reactor [Dodds, D.R., Lopez., J.L., Zepp, C.M., and
Rossi, R.F. PCT Patent Application No. WO 90/04643.
May, 1990].

The choice of which particular enzyme is best

20    for a given substrate pair is determined by treating
samples of the enantiomeric pairs with various enzymes
such as porcine liver esterase, porcine pancreatic
lipase, lipases from *Pseudomonas species* (PSL) and
lipase from *Aspergillus niger* (ANL), and proteases such

25    as subtilisin or α-chymotrypsin. After treatment of
the enantiomeric mixture with the resolving enzyme, the
products are isolated using standard extraction or
chromatography procedures. The enzyme producing the
greatest enantiomeric excess of the desired product

30    should be the best candidate for use in the process.

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The process can be further improved by
choosing a given enantiomeric mixture and resolving
enzyme combination and determining the ideal solvent
conditions for the reaction.  In a biphasic system, the

5   choice of organic solvent must be determined.  The
optimum organic solvent can be determined by treating
samples of the enantiomeric mixture with the selected
enzyme in the presence of the same amount of an array
of not more than about 50% water miscible organic

10  solvents.  Particular solvents include any not more
than about 50% water miscible (solubility less than 50%
in water at room temperature) alcoholic, alkane,
aromatic, ketone ether, nitro, halo-alkane or aromatic
organic solvents, such as n-amyl alcohol, iso-amyl

15  alcohol, tert-amyl alcohol, 3-pentanol, 1- or 3-
heptanol, 3-methyl-3-pentanol, 4-methyl-2-pentanol, 3-
ethyl-3-pentanol, 1- or 2-butanol, nitromethane,
dichloromethane, methyl isobutyl ketone, dimethyl
sulfide, sulfolane, etc.  Following treatment of an

20  enantiomeric mixture with the resolving enzyme in the
presence of equal amounts of various solvents, the
products are isolated using standard extraction or
chromatography procedures.  The solvent/enzyme pair
producing the greatest enantiomeric excess of the

25  desired product should be the best candidate for use in
the process.

The relative quantity of the selected organic
solvent should also be evaluated in order to achieve
the best results.  To do this, a similar procedure as

30  described above is followed.  Using a particular
enzyme/racemic mixture, the ratio of the selected
organic solvent/aqueous solvent is varied in a manner
such as the following: 95:5, 90:10, 80:20, 70:30,
60:40, 50:50, 40:60, 30:70, 20:80, 10:90 and 5:95,

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([organic solvent]: [aqueous solvent]).  Identical
samples of an enantiomeric mixture are treated with a
standard amount of a particular enzyme in the presence
of varying ratios of organic solvent to aqueous solvent
5  for a set time.  The total volume is kept constant.
Following treatment of an enantiomeric mixture with the
resolving enzyme in the presence of equal amounts of
various solvents, the products are isolated using
standard extraction or chromatography procedures.  The
10  solvent system/enzyme pair producing the greatest
enantiomeric excess of the desired product should be
the best candidate for use in the process.

Alternatively, for some racemic
mixture:enzyme: organic solvent combinations, enzyme
15  activity may be enhanced and organic solvent levels
reduced by adding surfactants to the reaction.  In
order to evaluate whether a surfactant should be added
to a particular process.  Some variation of the
following process may be pursued.  First, a surfactant
20  is selected by treating samples of an enantiomeric
mixture with the selected enzyme and an array of
surfactants in the presence of a non-homogeneous system
composed of a not more than about 50% water miscible
organic solvent and an aqueous solvent.  The system
25  should be one which is compatible with carrying out the
reaction in the absence of a surfactant.  Examples of
surfactants include the Tweens, such as Tween 20™,
Tween 80™, Prionex™, Teepol HB7™, Tergitol TMN-6™,
Tergitol TMN-10™, Tergitol NP-4™, Tergitol 15-S-3™,
30  Igepal CA-630™, Tyloxapol™, Glucode-oxycholic acid,
octyl β-gluco-pyranoside, CHAPS™, dioctyl
sulfosuccinate, or deoxycholic acid.  Following
treatment of an enantiomeric mixture with the resolving
enzyme in the presence of a biphasic solvent system and

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a constant amount of various surfactants, the products
are isolated using standard extraction or
chromatography procedures. The solvent/enzyme/
surfactant combination producing the greatest
5    enantiomeric excess of the desired product in a set
time should be the best candidate for use in the
process.

The surfactant may be added at a
concentration or range of concentrations depending on
10   how many samples can be processed at one time. For a
given solvent/enzyme/ surfactant combination, the
optimal surfactant concentration should be determined.
One of skill in the art will appreciate that an array
of independent reactions should be set up, differing
15   only by the concentration of surfactant. For example,
the reaction may be carried out using PLE in 20%
pentanol and 80% Tris(hydroxymethyl)aminomethane or [2-
amino-2-(hydroxymethyl)-1,3-propanediol buffer at pH
7.4. Ten identical reactions may be set up, having the
20   following surfactant concentrations: 1%, 3%, 5%, 7.5%
10%, 12.5%, 15%, 20%, 25% and 30%. Following treatment
of an enantiomeric mixture with the resolving enzyme in
the presence of a biphasic solvent system and
increasing surfactant concentration for a set time, the
25   products are isolated using standard extraction or
chromatography procedures. The solvent/enzyme/
surfactant combination producing the greatest
enantiomeric excess of the desired product in a set
time should be the best candidate for use in the
30   process.

Surfactants useful for carrying out this
invention include cationic, anionic, non-ionic or
amphoteric, or mixtures thereof. The preferred
surfactant will depend upon the particular enzyme

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and/or substrate components.  Such screening procedures
are well known to those of skill in the art.
Illustrative screening processes are set forth in
Examples 14-30.

5          Examples of useful cationic surfactants
include amines, amine salts, sulfonium, phosphonium and
quarternary ammonium compounds.  Specific examples of
such cationic surfactants include:
           Methyl trioctylammonium chloride
10         (Aliquat 336)
           N,N',N'-polyoxyethylene(10)-N-tallow-1,3-
diaminopropane
           (EDT-20,' PEG-10 tallow).
           Useful anionic surfactants include, for
15  example, linear alkylbenzene sulphonate, alpha-olefin
sulphonate, alkyl sulphate, alcohol ethoxy sulfate,
carboxylic acids, sulfuric esters and alkane sulfonic
acids.  Examples of anionic surfactants include:
           Triton QS-30 (Anionic)
20         Aerosol 22
           dioctyl sulfosuccinate (AOT)
           Alkyl Sodium Sulfate (Niaproof):
                Type-4
                Type-8
25              Alkyl (C9-C13) Sodium Sulfates
                (TEEPOL HB7).

           Non-ionic surfactants useful for
stabilization include nonyl phenol ethoxylate, alcohol
ethoxylate, sorbitan trioleate, non-ionic block
30  copolymer surfactants, polyethylene oxide or
polyethylene oxide derivatives of phenol alcohols or
fatty acids.  Examples of non-ionic surfactants
include:
           Polyoxyethylene Ethers:

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4 lauryl Ether (Brij 30)

23 lauryl Ether (Brij 35)

Octyl Phenoxy polyethoxyethanol (Tritons):

Tx-15

Tx-100

Tx-114

Tx-405

DF-16

N-57

DF-12

CF-10

CF-54

Polyoxyethylenesorbitan:

Monolaurate (Tween 20)

Sorbitan:

Sesquioleate (Arlacel 83)

Trioleate (Span 85)

Polyglycol Ether (Tergitol):

Type NP-4

Type NP-9

Type NP-35

Type TMN-10

Type 15-S-3

Type TMN-6(2,6,8, Trimethyl-4-nonyloxypolyethylenoxyethanol

Type 15-S-40.


After selecting a suitable surfactant, the ratio of organic solvent may sometimes be reduced significantly without losing product yield or enantioslectivity. One of skill in the art will appreciate that one such procedure for determining how much to lower the organic solvent is as follows: Using a particular enzyme/racemic mixture/surfactant

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combination the ratio of the selected organic solvent
to aqueous solvent is varied as follows: [% organic
solvent: % aqueous solvent], 95:5, 90:10, 80:20, 70:30,
60:40, 50:50, 40:60, 30:70, 20:80, 10:90 and 5:95, and

5    other ratios as required.  Samples of an enantiomeric
mixture are treated with a standard amount of a
particular enzyme in the presence of varying ratios of
organic solvent to aqueous solvent and surfactant for a
set time.  Following treatment of an enantiomeric

10   mixture with the resolving enzyme in the presence of
equal amounts of various solvents, the products are
isolated using standard extraction or chromatography
procedures.  The solvent/enzyme pair producing the
greatest enantiomeric excess of the desired product

15   should be the best candidate for use in the process.


An additional consideration for carrying out
the process of the present invention is the cost of the
enzyme per unit of product produced.  The present
invention is directed to reducing the enzyme

20   requirements of the process on a per unit of product
basis.  In one embodiment, the amount of organic
component is reduced in the non-homogeneous system.  In
another embodiment, a surfactant is added to the non-
homogeneous system to further reduce the amount of

25   enzyme required and further reduce the cost of
operating the process.
The present invention is particularly
directed to enzyme reactions wherein the substrate
comprises a hydrophobic ester.  The present invention

30   is additionally  directed to enzyme reactions wherein
the substrate is relatively insoluble in aqueous
solutions.  The use of a non-homogeneous system having
incompletely water miscible organic co-solvents

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provides improved solvation for hydrophobic esters and
other hydrophobic and insoluble compounds as compared
to systems using water miscible organic solvents.

     In order that this invention may be better
5  understood, the following examples are set forth.
These examples are for the purpose of illustration only
and are not to be construed as limiting the scope of
the invention in any matter.

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## EXAMPLES

**Example 1, Porcine Liver Esterase Catalyzed Resolution of FTC butyrate**

Racemic FTC-butyrate (1.0 g) was dissolved in 5.0
5   ml of n-amyl alcohol by heating to 75 °C for 30 minutes
to make an organic component. The organic component
was then mixed with an aqueous component comprising 3.8
ml of 0.3 M pH 7.5 phosphate buffer and the non-
homogeneous system was allowed to cool to 35 °C.
10   Porcine liver esterase solution, 1.2 ml of 650 U/ml
Altus PLE solution (Altus Biologics, Cambridge, MA) was
then added to the aqueous layer and the resulting
suspension was stirred with gentle agitation. The
temperature was maintained at 32 °C by an external
15   water-bath. The pH was maintained at 7.5 by the
addition of 50% aqueous sodium hydroxide as necessary.
The optical purity of the unreacted (-)-butyrate ester
and the (+)-FTC alcohol product were monitored by HPLC
analysis using a chiral stationary phase column. After
20   24 hours, the (+)-enantiomer of the FTC ester was
completely converted based on HPLC analysis as
described below. Extraction of the unreacted ester
from the organic phase and evaporation of the organic
solvent gave the desired (-)-FTC ester. The recovered
25   yield was 89.4 % based on the single (-) enantiomer and
the optical purity was greater than 99 %.

Procedures

Chiral HPLC conditions : CHIRAPAK® AS; 0.46
cm x 25 cm HPLC column (Daicel Chemical Inc.), mobile
30   phase = 100 % acetonitrile, flow rate = 1 ml/min., uv
detection at 260 nm. Retention times: (-)-FTC
butyrate, 6.2 min.; (-)-FTC, 7.4 min.; (+)-FTC
butyrate, 8.8 min.; and (+)-FTC, 11.4 min.

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Enzyme activity was determined by the
conversion of ethyl butyrate using a Radiometer pH-stat
apparatus to follow the production of acid.  Ethyl
butyrate (40 ml) was added to 20 ml of 5 mM boric acid
5       (pH 8) and stirred at 25°C until dissolution was
complete (10 minutes).  PLE was added and the pH was
maintained at 8.0 by the addition of 0.01 N NaOH.  The
rate of acid production was determined from the rate of
base addition over a period of 5 minutes.
10              Enzyme stability was measured while the
reaction was in progress.  Measurements were performed
by periodically removing aliquots of the enzyme
solution and determining the activity using the ethyl
butyrate assay.

15  **Example 2, CLEC™-PLE catalyzed reaction of FTC butyrate
in 83% of n-amyl alcohol (or 3-Me-3-pentanol)/aqueous
mixture**
The reaction conditions and procedures were
the same as in Example 1, except the volume of
20  phosphate buffer was 1 ml and the volume of the organic
component was 8.3 ml.  The conversion was 38% for n-
amyl alcohol and 25% for 3-methyl-3-pentanol after 36 h
(see Table 1, Reactions 12 and 13).

**Example 3, PSL-catalyzed reaction of FTC butyrate in
25  50% n-amyl alcohol/aqueous mixture**
The reaction conditions and procedures were
the same as in Example 1, except that 100 mg of soluble
PSL-30 (PSL-30 is PS30 from Amano) was used.  The
conversion was 56% after 24 h and the (-)-enantiomer
30  was preferentially hydrolyzed.  The optical purity of

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the remaining ester was 92% at 56% conversion (see
Table 1, Reaction 21).

**Example 4, ANL-catalyzed reaction of FTC butyrate in
50% n-amyl alcohol/aqueous mixture**

5          The reaction conditions and procedures were
the same as in Example 1, except that 200 mg of soluble
ANL was used.  The conversion was 45% after 36 h.  The
optical purity of the remaining ester was 63% at 45%
conversion (see Table 1, Reaction 22).

10   **Example 5, PLE-catalyzed conversion of (±)-FTC butyrate
in 20% of isopropanol (or other water-miscible organic
co-solvents)/aqueous mixture with 2% substrate
concentration**

          The following example illustrates the state
15   of the art using high amounts of enzyme catalysts in a
homogeneous system.  To a solution of 1 ml of Altus PLE
solution 650 units/ml from Altus Biologics, Inc. in 39
ml of 0.3 M phosphate buffer (pH 7.5) was added 10 ml
of 10% FTC butyrate in isopropanol.  The resulting

20   mixture was stirred at 24~26°C and the reaction
progress was monitored by HPLC.  The conversion reached
51% and the optical purity of the remaining chiral non-
racemic ester compound was greater than 99% (48%
chemical yield) after a 22 h reaction.  These results

25   are based on HPLC analysis of the remaining chiral non-
racemic ester compound.  The aqueous layer included
hydrolyzed products (+)-FTC and (-)-FTC.  The ratio of
(+)-FTC and (-)-FTC was 96.6 to 3.4.  The organic layer
was evaporated to give 0.457 g of (-)-FTC butyrate.

30          A similar reaction was performed by using
other  water miscible organic co-solvents, including

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acetonitrile, DMF, 1-methyl-2-pyrrolidinone, methanol,
ethanol, *tert*-butanol, DMSO, pyridine, di(ethylene
glycol)methyl ether, PEG 200, and PEG 600 etc.
Acetonitrile gave the same high enantioselectivity as
5   isopropanol and required similarly large amounts of
enzyme.  All other solvents gave lower
enantioselectivity than isopropanol.


**Example 6, PLE-catalyzed conversion of (±)-FTC butyrate
in 20% of isopropanol/aqueous mixture with 5% substrate
10  concentration**

To a solution of 2.5 ml of Altus PLE solution
650 units/ml from Altus Biologics, Inc. in 37.5 ml of
0.3 M phosphate buffer (pH 7.5) was added 10.0 ml of
25% FTC butyrate in isopropanol.  Under these
15  conditions, the substrate was incompletely dissolved.
The resulting mixture was stirred at 24~26°C and the
reaction was monitored by HPLC.  The conversion reached
60% and the optical purity of the remaining ester was
74% (38% chemical yield) after 96 h reaction time.  The
20  enantioselectivity was much lower than the reaction
with a 2% substrate concentration.


**Example 7, PLE-catalyzed conversion of (±)-FTC butyrate
in 30% isopropanol/aqueous solution and a 3% substrate
concentration**
25          To a solution of 1.5 ml of Altus PLE solution
650 units/ml from Altus Biologics, Inc. in 33.5 ml of
0.3 M phosphate buffer (pH 7.5) was added 15 ml of 10%
FTC butyrate in isopropanol.  The resulting mixture was
stirred at 24~26°C and the reaction was monitored by
30  HPLC.  The conversion was 8% after 2 h and did not

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increase after that.  The enzyme rapidly lost all
activity in the 30% isopropanol.

　　　　Table 1 summarizes the results of resolution
reactions of FTC-butyrate with various enzymes in
5　biphasic non-homogeneous systems comprising various not
more than about 50% water miscible organic solvents and
aqueous buffer solutions.

Table 1: Resolution of an enantiomeric mixture of FTC-
butyrate with various enzymes in various biphasic
10　systems[a]

| Rxn | enzyme | co-organic solvent | time (h) | conver-sion(%) | ee(%)[b] ester | stereochem preference |
|-----|--------|--------------------|----------|-----------------|------------------|-----------------------|
| 1 | PLE-C[c] | n-amyl alcohol | 24 | 52 | >98 | (+) |
| 2 | PLE-I[c] | n-amyl alcohol | 36 | 53 | >98 | (+) |
| 3 | PLE-S[c] | n-amyl alcohol | 24 | 52 | >98 | (+) |
| 4 | PLE-C | iso-amyl alcohol | 36 | 52 | >98 | (+) |
| 5 | PLE-C | tert-amyl alcohol | 36 | 37 | 59 | (+) |
| 6 | PLE-C | 1-butanol | 24 | 12 | 10 | (+) |
| 7 | PLE-C | 2-butanol | 24 | 7 | 7.5 | (+) |
| 8 | PLE-C | 3-pentanol | 36 | 40 | 67 | (+) |
| 9 | PLE-C | 1-heptanol | 36 | 39 | 64 | (+) |
| 10 | PLE-C | 3-heptanol | 36 | 39 | 52 | (+) |
| 11 | PLE-C | 3-Me-3-pentanol | 36 | 53 | >98 | (+) |
| 12 | PLE-C | 3-Me-3-pentanol[d] | 36 | 25 | 33 | (+) |
| 13 | PLE-C | n-amyl alcohol[d] | 36 | 38 | 61 | (+) |
| 14 | PLE-C | 4-Me-2-pentanol | 36 | 45 | 82 | (+) |
| 15 | PLE-C | 3-Et-3-pentanol | 36 | 48 | 92 | (+) |
| 16 | PLE-C | nitromethane | 36 | 24 | 32 | (+) |
| 17 | PLE-C | dichloromethane | 36 | 20 | 25 | (+) |

| 18 | PLE-C | toluene | 36 | 18 | 16 | (+) |
|----|-------|---------|----|----|----|----|
| 19 | PLE-C | methyl isobutyl ketone | 36 | 20 | 33 | (+) |
| 20 | PLE-C | tert-butyl acetate | 36 | 23 | 29 | (+) |
| 21 | PSL | n-amyl alcohol | 24 | 56 | 92 | (-) |
| 22 | ANL | n-amyl alcohol | 36 | 45 | 63 | (+) |

a. Reaction conditions: 1g of (±)FTC-butyrate in 10 ml of 50% organic/aqueous mixture was hydrolyzed with PLE, PSL or ANL at room temperature.  b. The optical purity was based on HPLC analysis.  c. PLE-C = CLEC™-PLE, PLE-I = immobilized PLE, PLE-S = Altus PLE solution 650 units/ml.  d. in 6 ml or 83% organic/aqueous mixture.

Examples 5-7 illustrate some of the problems with using water miscible alcohols in homogeneous systems for the process of the present invention.  Such systems produce product with reduced optical purity, prolong reaction times, and deactivate the enzyme.

**Examples 8-13, PLE Catalyzed Conversion of (+) FTC-Butyrate in Non-Homogeneous Systems using n-Amyl Alcohol and Water.**

The reaction conditions and procedures were the same as in Example 1.  The non-homogeneous system comprises  1 ml of Altus PLE solution (650 U/ml) as catalyst and the volumes of amyl alcohol and phosphate buffer used are indicated in Table 2 below.  Note that in each case, the selectivity of conversion of the (+)-isomer was almost absolute, so that the desired conversion of slightly greater than 50% results in

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enantiomeric purities of the unreacted (-) ester of
nearly 100% (See Table 2).

Table 2, Examples 8 through 13

| Example | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|
| Amyl Alcohol | 2 ml | 3 ml | 4 ml | 5 ml | 6 ml | 7 ml |
| Phosphate buffer | 7 ml | 6 ml | 5 ml | 4 ml | 3 ml | 2 ml |
| Reaction time (h) | % Conversion | | | | | |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1 | 28 | 26 | 24 | 21 | 20 | 19 |
| 3 | 43.2 | 42 | 39 | 34 | 33 | 33 |
| 8 | 49 | 49 | 48 | 45 | 41 | 41 |
| 24 | 49.8 | 49.8 | 49.2 | 47.5 | 46.7 | 47.3 |

Examples 14-30, PLE-Catalyzed Conversion of (±) FTC-
Butyrate in non-homogeneous systems comprising n-amyl
alcohol and water mixtures in the presence of
surfactants.

Examples 14 through 30 are shown in Table 3.
The reaction conditions and procedures were the same as
in Example 1.  The non-homogeneous system comprises 1
ml of Altus PLE solution (650 U/ml) as catalyst and 1
ml (Examples 14-21, 23, 24, and 30) or 0.1 g of
surfactant (Examples 25-29) were added as surfactant to
the reaction mixtures.  The organic component comprised
n-amyl alcohol and the aqueous component comprised 0.3
M phosphate buffer in a 50:50 ratio.

Table 3, Examples 14 through 30

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| Example | Surfactant | % Conversion at time (t) | | | | |
|---|---|---|---|---|---|---|
| | | (t) Hours | | | | |
| | | 0 | 1 | 3 | 7 | 24 |
| 14 | Tween 20 | 0 | 18 | 34 | 45 | 50 |
| 15 | Prionex | 0 | 17 | 30 | 42 | 49 |
| 16 | Teepol HB7 | 0 | 9 | 15 | 21 | 26 |
| 17 | Tergitol TMN-6 | 0 | 14 | 32 | 44 | 48 |
| 18 | Tergitol 15-S-3 | 0 | 17 | 29 | 40 | 47 |
| 19 | Igepal CA-630 | 0 | 19 | 35 | 45 | 49 |
| 20 | Tyloxapol | 0 | 18 | 35 | 46 | 50 |
| 21 | Tergitol TMN-10 | 0 | 17 | 30 | 42 | 48 |

Table 3 continued

| Example | Surfactant | % Conversion at time (t) | | | |
|---|---|---|---|---|---|
| | | (t) Hours | | | |
| | | 0 | 1 | 3 | 20 |
| 22 | No Surfactant | 0 | 21 | 34 | 47.5 |
| 23 | Aerosol 22 | 0 | 7 | 7 | 8 |
| 24 | Tergitol NP-4 | 0 | 18 | 34 | 49.5 |
| 25 | Glucode-oxycholic acid | 0 | 14 | 25 | 44 |
| 26 | Octyl β-gluco-pyranoside | 0 | 15 | 30 | 47 |
| 27 | CHAPS | 0 | 14 | 21 | 39 |
| 28 | Dioctyl Sulfo-succinate Na+ salt | 0 | 17 | 32 | 49.5 |
| 29 | Deoxy-cholic acid Na+ salt | 0 | 13 | 23 | 43.4 |
| 30 | Tween 80 | 0 | 18 | 33 | 50 |

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The broad screening of surfactants, as shown
in Table 3, reveals that some are activating (see
Examples 14, 15, 19, 20, 24, 28, and 30) and some are
inhibitory (see 16, 23, 27 and 29).  Fifteen
5   surfactants were chosen for further analysis.  The
surfactants Tergitol NP-4, Tween 80, Tyloxapol and
dioctyl sulfosuccinate sodium all enhanced the PLE
activity to roughly the same extent.  The enhancement
in rate is most apparent at the end of the reaction and
10  may be due to stabilization of the enzyme and
prevention of precipitation as well as an effect on
catalytic efficiency.

**Example 31-34, PLE Catalyzed Conversion of (±) FTC-
Butyrate in Bi-phasic n-Amyl Alcohol/Water mixtures in
15  the presence of Tween-80.**

Examples 31 through 34 are shown in Table 4.
The reaction conditions and procedures were the same as
in Example 1.  The non-homogeneous system comprises
Tween 80 as surfactant, 0.6 ml Altus PLE solution (650
20  U/ml) as the catalyst and the volume of amyl alcohol
and 0.3 M phosphate buffer used are indicated in the
table below.

**Table 4, Examples 31 through 34**

| Example | 31 | 32 | 33 | 34 |
|---|---|---|---|---|
| Amyl Alcohol | 4 ml | 4.5 ml | 4.75 ml | 4.9 ml |
| Tween-80 | 1 ml | 0.5 ml | 0.25 ml | 0.1 ml |
| Phosphate buffer 0.3M | 5 ml | 5 ml | 5 ml | 5 ml |
| Reaction time (h) | 1 | 1 | 1 | 1 |
| % Conversion | 10 | 8 | 6 | 5 |

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**Examples 35, Complimentary reductions in both enzyme and organic solvent requirements**

The reaction conditions and procedures were the same as in Example 1. The non-homogeneous system
5    comprised  0.5 ml of Tween 80 as surfactant, 0.3 ml of Altus PLE solution (650 U/ml) as catalyst, and 2.0 ml of amyl alcohol and 7.5 ml of 0.3 M phosphate buffer were the solvents. The non-homogeneous system comprised 25% organic component and 75% aqueous
10   component. After 48 hours, the extent of conversion was 50% and the optical purity of the remaining ester was 99.3%.

In this example, the amount of both enzyme and organic solvent were reduced by approximately half
15   from the level used in Examples 31-34, with no loss of product yield. Furthermore, the enzyme requirement was only 25% of that required in Example 1.


**Examples 36-39, Dioctyl sulfosuccinate (Dioctyl SS) as surfactant.**

20       Examples 36 through 39 are shown in Table 5. The reaction conditions and procedures were the same as in Example 1. The non-homogeneous system comprised dioctyl sulfosuccinate (Dioctyl SS) as surfactant and 0.4 ml of Altus PLE enzyme solution (650 U/ml) in 8 ml
25   of 0.3 M phosphate buffer and 2 ml of amyl alcohol.

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Table 5, Examples 36 through 39

| Example | 36 | 37 | 38 | 39 |
|---------|-----|-----|-----|-----|
| Dioctyl SS | 10 mg | 25 mg | 100 mg | 200 mg |
| Time(h) | Conversion | | | |
| 0 | 0 | 0 | 0 | 0 |
| 1 | 5 | 5.5 | 9 | 9 |
| 3 | 20 | 22 | 25 | 23 |
| 5.5 | 27 | 32 | 34 | 30 |
| 21 | 40 | 47 | 48 | 45 |

10   Example 40

The reaction conditions and procedures were
the same as in Example 1.  The catalyst comprised 714
total units of porcine liver esterase (Sigma, st.
Louis, MO).  The non-homogeneous system comprised 50%
15   n-amyl alcohol as organic component and 50% 0.3 M
phosphate buffer at pH 7.4 as aqueous component.  After
24 hours, the extent of conversion was 50% and the
optical purity of the remaining ester was 97.5%.


Example 41, Rate enhancement with low enzyme loadings
20   and anionic surfactant

In addition to the use of Tween-80, the
anionic surfactant dioctyl sulfosuccinate sodium salt,
was chosen to achieve rate enhancement.  As shown in
Table 6, a 1% loading of this surfactant in the non-
25   homogeneous system was sufficient for significant rate
enhancement.

Reaction conditions included: 1 g FTC
butyrate, 0.4% PLE loading, organic solvent 1-pentanol,
2:8 solvent ratio, reaction carried out at 30 °C (Table
30   6).

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Table 6, Example 41

| | % Conversion with (x mg) Surfactant | | | |
|---|---|---|---|---|
| Time (h) | mg surfactant | | | |
| | 10 mg | 25 mg | 100 mg | 200 mg |
| 0 | 0 | 0 | 0 | 0 |
| 1 | 5 | 5.5 | 9 | 9 |
| 3 | 20 | 22 | 25 | 23 |
| 5.5 | 27 | 32 | 34 | 30 |
| 21 | 40 | 47 | 48 | 45 |

**Example 42, Surfactant effect on enzyme loading and organic solvent concentration**

A preferred embodiment of this invention includes an enzyme loading of 0.3 to 0.4 % relative to FTC butyrate with a 10% substrate loading. A number of reactions were performed on slightly larger scale to more accurately determine the run to run variation and the effect of conversion on optical purity. The results are shown in Table 7 below.

Table 7, 5 g Scale Reactions at Low Enzyme Loadings (28 °C, 45% 1-pentanol, 5% Tween-80, 50% aqueous)

| PLE (%) | Tween-80 (%) | Time (h) | Optical Purity (% e.e.) |
|---|---|---|---|
| 0.6 | 2.5 | 26 | 95.32 |
| 0.6 | 5 | 24 | 98.34 |
| 0.4 | 5 | 24 | 96.20 |
| 0.4 | 5 | 42 | >99.0 |

As shown in Table 8, reactions performed at a lower organic/aqueous ratio and with a 0.3% enzyme loading gave high optical purity in less than 48 hours.

Table 8. 1 g Scale Reaction at Low Enzyme Loadings, (28 °C in 20% 1-pentanol/5% Tween-80, 75% aqueous)

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| PLE (%) | Time (h) | e.e. (%) |
|---------|----------|----------|
| 0.4 % | 25 | 99.20 |
| 0.3 % | 25 | 95.88 |
| 0.3 % | 31 | 97.68 |
| 0.3 % | 48 | 99.32 |

While we have hereinbefore described a number of embodiments of this invention, it is apparent that our basic constructions can be altered to provide other embodiments which utilize the processes and compositions of this invention. Therefore, it will be appreciated that the scope of this invention is to be defined by the claims appended hereto rather than by the specific embodiments which have been presented hereinbefore by way of example.

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CLAIMS

We Claim:

1.   A process for producing a chiral, non-racemic ester of Formula I using a hydrolase enzyme:
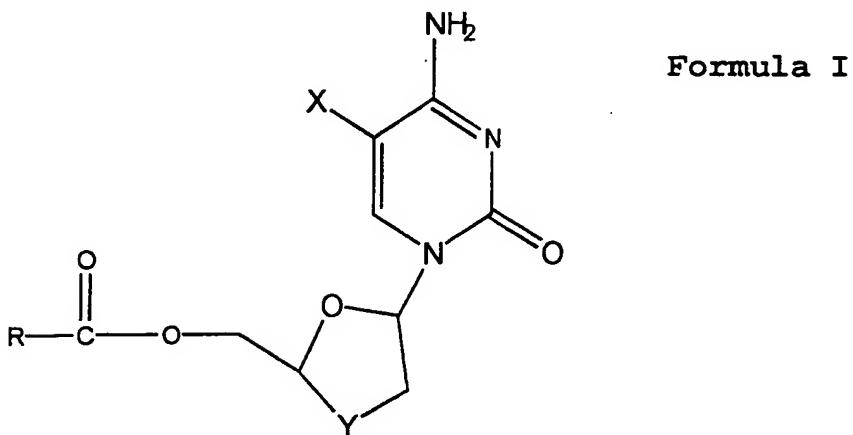


**Formula I**

wherein:
R is $C_1$-$C_8$ alkyl, alkenyl, or alkynyl;
X = H, or F;
Y = $CH_2$, O, S, Se, or NH;
said process comprising the steps of:
(a) dispersing an enantiomeric mixture of an ester of Formula I at a concentration of between about 1 and about 25% (weight/volume of the non-homogeneous system), in an organic solvent system to produce an organic component;
(b) providing an aqueous solvent system to produce an aqueous component; and
(c) contacting said organic component and said aqueous component to form a non-homogeneous system, under conditions which permit the resolution of the mixture to produce a chiral non-racemic ester of Formula I and a non-racemic alcohol of Formula II;

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Formula II
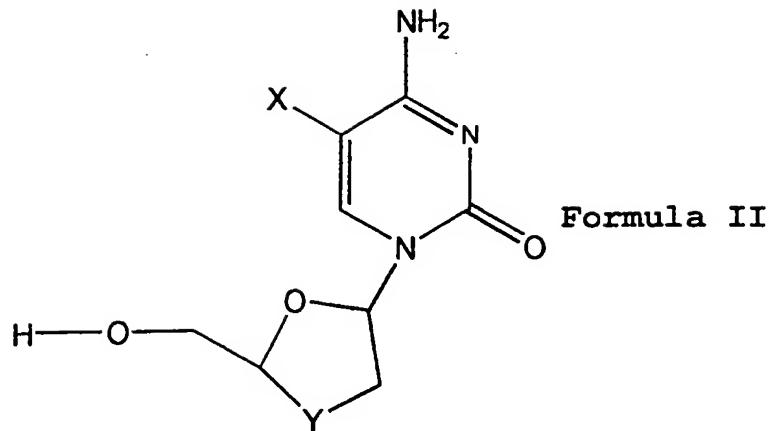
wherein:

X = H, or F;

Y = CH$_2$, O, S, Se, or NH, and

5        wherein said hydrolase enzyme is dispersed in either said organic component, said aqueous component or said non-homogeneous system.


        2.    A process for producing a chiral, non-
10  racemic hydrophobic ester using a hydrolase enzyme, said process comprising the steps of:

        (a) dispersing an enantiomeric mixture of said hydrophobic ester at a concentration of between about 1 and about 25% (weight/volume of the non-
15  homogeneous system), in an organic solvent system to produce an organic component;

        (b) providing an aqueous solvent system to produce an aqueous component; and

        (c) contacting said organic component and
20  said aqueous component to form a non-homogeneous system, under conditions which permit the enantioselective conversion of one enantiomeric form of said enantiomeric mixture to the corresponding alcohol; and

25        wherein said hydrolase enzyme is dispersed in either said organic component, said aqueous component or said non-homogeneous system.

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3.    A process for producing a chiral, non-racemic ester of 2-butyryloxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane using a hydrolase enzyme, said process comprising the steps of:

5           (a) dispersing an enantiomeric mixture of said 2-butyryloxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane at a concentration of between about 1 and about 25% (weight/volume of the non-homogeneous system), in an organic solvent system to produce an
10   organic component;

(b) providing an aqueous solvent system to produce an aqueous component; and

.(c) contacting said organic component and said aqueous component to form a non-homogeneous
15   system, under conditions which permit the enantioselective conversion of one enantiomeric form of said enantiomeric mixture to the corresponding alcohol;

wherein said hydrolase enzyme is dispersed in either said organic component, said
20   aqueous component or said non-homogeneous system; and

wherein the concentration of said enantiomeric mixture is calculated based on the volume of said non-homogeneous system.

4.    A process for producing a chiral, non-
25   racemic ester of 2-butyryloxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane using a hydrolase enzyme, said process comprising the steps of:

(a) dispersing an enantiomeric mixture of said 2-butyryloxymethyl-5-(5-fluorocytosin-1-yl)-1,3-
30   oxathiolane at a concentration of between about 1 and about 25% (weight/volume of the non-homogeneous system), in an organic solvent system to produce an organic component;

(b) providing an aqueous solvent system to
35   produce an aqueous component; and

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(c) contacting said organic component and said aqueous component to form a non-homogeneous system, under conditions which permit the enantioselective conversion of one enantiomeric form of

5   said enantiomeric mixture to the corresponding alcohol;

wherein said hydrolase enzyme is dispersed in either said organic component, said aqueous component or said non-homogeneous system;

wherein said organic component comprises

10  between about 5 and about 90% of said non-homogeneous system;

wherein said non-homogeneous system also comprises between about 1 and about 20% of surfactant; and

15            wherein said surfactant concentration is calculated based on the volume of said non-homogeneous system.

5.    The process according to any one of claims 1, 2, 3 or 4, wherein said hydrolase enzyme is

20  selected from the group consisting of porcine liver esterase, porcine pancreatic lipase, *Pseudomonas species* lipase, *Aspergillus niger* lipase and subtilisin.

6.    The process according to claim 5,

25  wherein said hydrolase enzyme is a crosslinked enzyme crystal.

7.    The process according to claim 6, wherein said crosslinked enzyme crystal is crosslinked with glutaraldehyde.

30            8.    The process according to claim 5, wherein said hydrolase enzyme is an immobilized enzyme.

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9.    The process according to claim 5,
wherein said hydrolase enzyme is a soluble enzyme.

10.    The process according to claim 5,
wherein said hydrolase enzyme is porcine liver
5    esterase.

11.    The process according to any one of
claims 1, 2, 3 or 4, wherein said chiral non-racemic
ester is isolated from said organic component.

12.    The process according to any one of
10    claims 1, 2, 3 or 4, wherein said chiral non-racemic
alcohol is isolated from said aqueous component.

13.    The process according to any one of
claims 1 or 2, wherein said enantiomeric mixture is FTC
butyrate.

15          14.    The process according to claim 2,
wherein said enantiomeric mixture comprises 2-
butyryloxymethyl-5-(5-fluorocytosin-1-yl)-1,3-
oxathiolane.

15.    The process according to any one of
20    claims 1, 2, 3 or 4, wherein said enantiomeric mixture
is dispersed in said organic component to a
concentration of between about 5% to about 15%.

16.    The process according to any one of
claims 1, 2, 3 or 4, wherein said enantiomeric mixture
25    is dispersed in said organic component to a
concentration of between about 1% to about 5%.

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17.    The process according to any one of
claims 1 or 2, wherein said enantiomeric mixture is
dispersed in said organic component to a concentration
of between about 10% to about 20%.

5              18.    The process according to any one of
claims 1, 2, 3 or 4, wherein said organic component
comprises a not more than about 50% water miscible
organic solvent.

                19.    The process according to claim 18,
10    wherein said organic component comprises one or more
solvents selected from the group consisting of $C_4$-$C_8$
alcohols, nitromethane, dichloromethane, toluene,
methyl isobutyl ketone, tert-butyl acetate and alkanes.

                20.    The process according to claim 19,
15    wherein said organic component comprises one or both of
n-amyl alcohol and 3-methyl-3-pentanol.

                21.    The process according to claim 4,
wherein said surfactant is selected from the group
consisting of cationic surfactants, anionic surfactants
20    and non-ionic surfactants.

                22.    The process according to claim 21,
wherein said surfactant is selected from the group
consisting of Tween 20™, Tween 80™, Prionex™, Teepol
HB7™, Tergitol TMN-6™, Tergitol TMN-10™, Tergitol NP-
25    4™, Tergitol 15-S-3™, Igepal CA-630™, Tyloxapol™,
Glucode-oxycholic acid, octyl β-gluco-pyranoside,
dioctyl sulfosuccinate, and deoxycholic acid.

                23.    The process according to claim 22,
wherein said surfactant is Tween-80™.

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24. The process according to claim 22,
wherein said surfactant is dioctyl sulfosuccinate.

25. The process according to claim 4,
wherein said surfactant is added to said organic
5   component.

26. The process according to claim 4,
wherein said surfactant is added to said aqueous
component.

27. The process according to claim 4,
10  wherein said surfactant is added to said non-
homogeneous system.

28. The process according to claim 4,
wherein said surfactant is formulated with said
hydrolase enzyme.

15          29. The process according to any one of
claims 1, 2, 3 or 4, wherein said aqueous solvent
system comprises water and excipients selected from the
group consisting of buffering salts, alkalizing agents,
anti-microbial preservatives, stabilizers, filtering
20  aids, co-enzymes, excipients that facilitate dispersion
and excipients that facilitate function of the enzyme.

30. The process according to claim 29,
wherein said aqueous solvent system comprises water
buffered with phosphate buffer at a pH of greater than
25  about 7.

31. The process according to claim 29,
wherein said aqueous solvent system comprises water
buffered with 2-amino-2-(hydroxymethyl)-1,3-propanediol
or TRIS™.

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32.  The process according to any one of claims 1, 2, 3 or 4, wherein said conditions which permit the enantioselective conversion of one enantiomeric form of said enantiomeric mixture to the

5  corresponding alcohol comprise a temperature of between about 5°C and about 45°C.

33.  A non-homogeneous system for producing a chiral, non-racemic hydrophobic ester using a hydrolase enzyme, comprising:

10          (a) a hydrolase enzyme;
           (b) a hydrophobic ester substrate;
           (c) an organic component; and
           (d) an aqueous component.

34.  The non-homogeneous system according to

15  claim 33, wherein said hydrolase enzyme is selected from the group consisting of porcine liver esterase, porcine pancreatic lipase, *Pseudomonas species* lipase, *Aspergillus niger* lipase and subtilisin.

35.  The non-homogeneous system according to

20  claim 33, wherein said hydrolase enzyme is a crosslinked enzyme crystal.

36.  The non-homogeneous system according to claim 35, wherein said crosslinked enzyme crystal is crosslinked with glutaraldehyde.

25          37.  The non-homogeneous system according to claim 33, wherein said hydrolase enzyme is an immobilized enzyme.

38.  The non-homogeneous system according to claim 33, wherein said hydrolase enzyme is a soluble

30  enzyme.

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39. The non-homogeneous system according to claim 34, wherein said hydrolase enzyme is porcine liver esterase.

40. The non-homogeneous system according to claim 33, wherein said hydrophobic ester substrate is an enantiomeric mixture.

41. The non-homogeneous system according to claim 40, wherein said enantiomeric mixture comprises 2-butyryloxymethyl-5-(5-fluorocytosin-1-yl)-1,3-oxathiolane.

42. The non-homogeneous system according to claim 40, wherein said enantiomeric mixture is dispersed in said organic component to a concentration of between about 5% to about 15%.

43. The non-homogeneous system according to claim 40, wherein said enantiomeric mixture is dispersed in said organic component to a concentration of between about 10% to about 20%.

44. The non-homogeneous system according to claim 40, wherein said enantiomeric mixture is dispersed in said organic component to a concentration of between about 1% to about 5%.

45. The non-homogeneous system according to claim 33, wherein said organic component comprises a not more than about 50% water miscible organic solvent.

46. The non-homogeneous system according to claim 45, wherein said not more than about 50% water miscible organic solvent comprises one or more solvents selected from the group consisting of $C_4$-$C_8$ alcohols,

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nitromethane, dichloromethane, toluene, methyl isobutyl
ketone, tert-butyl acetate and alkanes.

47.    The non-homogeneous system according to
claim 46, wherein said organic component comprises one
5  or both of n-amyl alcohol and 3-methyl-3-pentanol.

48.    The non-homogeneous system according to
claim 33, further comprising a surfactant.

49.    The non-homogeneous system according to
claim 48, wherein said surfactant is selected from the
10  group consisting of cationic surfactants, anionic
surfactants and non-ionic surfactants.

50.    The non-homogeneous system according to
claim 49, wherein said surfactant is selected from the
group consisting of Tween 20™, Tween 80™, Prionex™,
15  Teepol HB7™, Tergitol TMN-6™, Tergitol TMN-10™,
Tergitol NP-4™, Tergitol 15-S-3™, Igepal CA-630™,
Tyloxapol™, Glucode-oxycholic acid, octyl β-gluco-
pyranoside, dioctyl sulfosuccinate, or deoxycholic
acid.

20         51.    The non-homogeneous system according to
claim 50, wherein said surfactant is Tween-80™.

52.    The non-homogeneous system according to
claim 50, wherein said surfactant is dioctyl
sulfosuccinate.

25         53.    The non-homogeneous system according to
claim 48, wherein said organic component comprises said
surfactant.

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54.  The non-homogeneous system according to claim 48, wherein said aqueous component comprises said surfactant.

55.  The non-homogeneous system according to
5  claim 48, wherein said surfactant is formulated with said hydrolase enzyme.

56.  The non-homogeneous system according to claim 33, wherein said aqueous solvent system comprises water and excipients selected from the group consisting
10  of buffering salts, alkalizing agents, anti-microbial preservatives, stabilizers, filtering aids, co-enzymes, excipients that facilitate dispersion and excipients that facilitate function of the enzyme.

57.  The non-homogeneous system according to
15  claim 33, wherein said aqueous solvent system comprises water buffered with phosphate buffer at a pH of greater than about 7.

58.  The non-homogeneous system according to claim 33, wherein said aqueous component comprises
20  water buffered with 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS™) at a pH of greater than about 7.

59.  The non-homogeneous system according to claim 33, wherein said organic component and said aqueous component are contacted under conditions which
25  permit the enantioselective conversion of one enantiomeric form of said enantiomeric mixture to the corresponding alcohol.

60.  The non-homogeneous system according to claim 59, wherein said organic component and said
30  aqueous component are contacted under conditions which permit the enantioselective conversion of one

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enantiomeric form of said enantiomeric mixture to the
corresponding alcohol, comprise a temperature of
between about 5°C and about 45°C.

# INTERNATIONAL SEARCH REPORT

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 7    C12P41/00    C12P19/02    C12P7/62    C07H19/06

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC 7    C12P    C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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| X | Further documents are listed in the continuation of box C. | | X | Patent family members are listed in annex. |
|---|---|---|---|---|

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 2 February 2000 | 16/02/2000 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016 | Douschan, K |

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